STRUCTURE and FUNCTION of CONNECTIVE and SKELETAL TISSUE

5.2 EXPERIENCE ON THE STARCH GEL ELECTROPHORESIS OF HEAT-DENATURED COLLAGENS

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During the work on the starch gel electrophoresis of heat-denatured collagens (Näntö, Maatela and Kulonen, 1963) we observed that the experimental details were critical for the optimal resolution, and the useful ranges were narrow and mutually interdependent. The length of the denaturation and the temperature during the run deserved special attention.

MATERIALS AND EQUIPMENT

The experimental details will be reported in full elsewhere (Näntö *et al.*, 1965). In the experiments on the optimal conditions the material was heat-denatured (15 min at 40°C) purified, neutral salt-soluble collagen of guinea-pig skin in 0·01 M, pH 4·8, acetate buffer. For the experiments on the length of the denaturation and on the temperature during the run purified, acetic acid-soluble collagen of rat tail tendon was used. If not specified, the electrophoresis was carried out at 38°C in a temperature-controlled room.

The gel was supported in a plastic trough and the ends of the gel were dipping without bridges in the electrode compartments, which were adopted from Kohn (1957).

GEL CONCENTRATION

The migration of the fastest band was inversely proportional to the gel concentration, which is in agreement with the general findings of Smithies

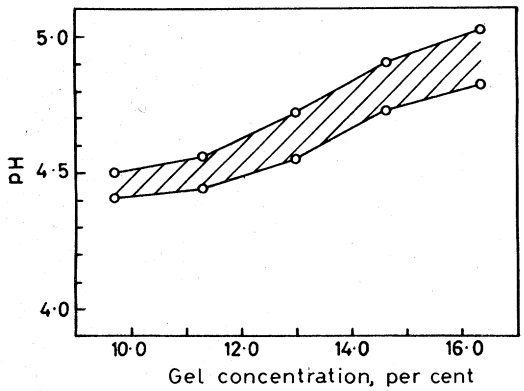

Figure 1. Effect of the gel concentration on the pH range of the optimal electrophoretic resolution of denatured salt-soluble collagen (shaded). The ionic strength $\mu = 0{\cdot}017$

(1962). At suitable pH and ionic strength an acceptable resolution was achieved with all the gel concentrations from 9·7 to 21·0 g% (*Figure 1*). In low gel concentrations the bands were slightly blurred because of diffusion, and the optimal resolution was obtained at lower pH levels. The

slow bands $x1$ and $x2$ (presumably higher aggregates) were especially retarded in very concentrated gels and at low pH.

The range of 14–15 g% was the most suitable compromise for the gel concentration. The bands were sharp, the useful pH range wide, the duration of the run within practical limits and the gel was not too viscous to be cast.

HYDROGEN ION CONCENTRATION

The best resolution was obtained in the pH range 4–5 (*Figure 2b*), at the gel concentration of 14·7%g at pH 4·73–4·87 with acetate buffer ($\mu = 0{\cdot}017$). For identification of the bands, see *Figure 3*.

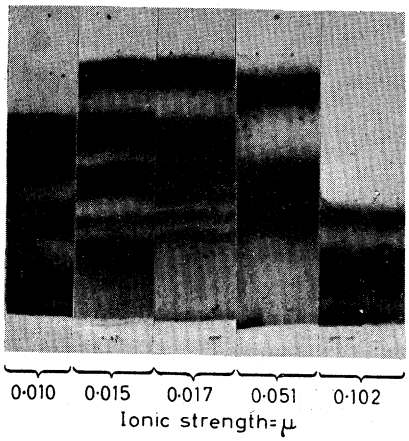

Figure 2a. Effect of the ionic strength inside the gel on the electrophoretic pattern of denatured salt-soluble collagen. Conditions: gel concentration 14·7 g%, pH 4·77, voltage gradient 5·8 V/cm, duration 3·5 hours

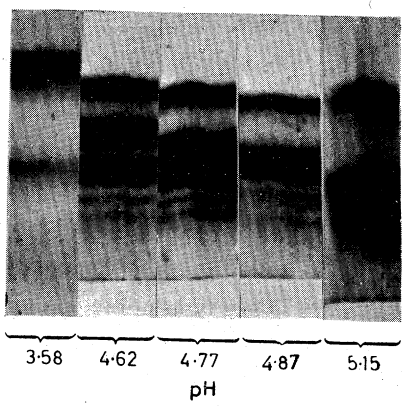

Figure 2b. Effect of pH on the electrophoretic pattern of denatured salt-soluble collagen. Ionic strength $\mu = 0{\cdot}022$

Figure 3 presents the absolute migration of the different subunits. The electrophoretic patterns vary with the pH and the composition of the bands varies as observed also from *Figure 2b*. The bands designated $\alpha1$ and $\beta2$ ($= \alpha1$–$\alpha1$) differ from the others, i.e., $\alpha2$, $\beta1$ ($= \alpha1$–$\alpha2$), $x1$ and $x2$ (various aggregates of tropocollagen), and these bands, which contain $\alpha1$-chains only, seem to have a more acid isoelectric point. According to the data of Piez, Eigner and Lewis (1963) the $\alpha1$-unit of rat skin and tail tendon collagen is more acid than the $\alpha2$-unit.

The most critical of all the studied variables was the ionic strength in the buffer which was used in the preparation of the gel (*Figure 2a*). The range of $\mu = 0{\cdot}017$–$0{\cdot}022$ was the most favourable.

The charge of collagen depends on the ionic strength of the environment (Jackson and Neuberger, 1957). At higher ionic strength the apparent isoelectric point is lower. Our results agree, i.e., when the ionic strength is

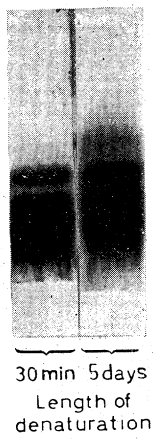

Figure 2c. Demonstration of a fast-migrating band after denaturation of acetic acid-soluble collagen for 5 days at 40°C. In the part on the right the sample has been increased to accentuate the fast band

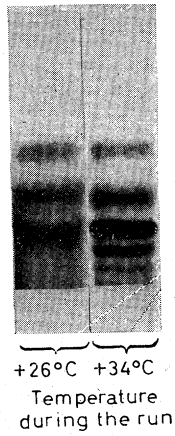

Figure 2d. Effect of the temperature during the run on the electrophoretic pattern of denatured acetic acid-soluble collagen

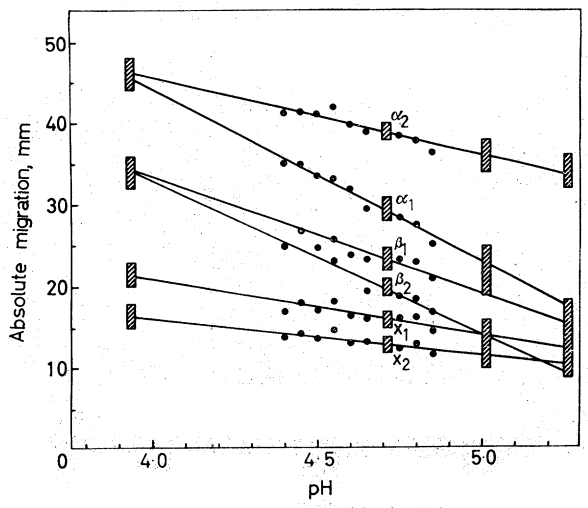

Figure 3. Effect of pH on the absolute migration of the various bands from denatured salt-soluble collagen. Conditions: gel concentration 14·7 g%, ionic strength $\mu = 0{\cdot}022$, voltage gradient 5·8 V/cm, duration 3·5 hours

increased, the effect is the same as making the pH more alkaline. The subunits are not equally sensitive to the variations in the ionic strength, and the sequence and composition of the bands change when the ionic strength is altered.

No change was observed in the resolution or in the mutual relationships of the fractions, when the salt concentration was higher in the electrode compartments than inside the gel. The final gel temperature and the migration rates increased.

VOLTAGE GRADIENT, GEL TEMPERATURE AND DURATION

With a voltage gradient of 8·7 V/cm the temperature rose to 55°C inside the gel and the current increased from 24 to 34 mA during the usual run of 2·5 hours (ionic strength $\mu = 0{\cdot}017$). At higher voltages the temperature increased so much that the viscosity of the gel decreased and the bands became hazy.

The question arises as to which is the safe limit of temperature in the gel to avoid eventual decomposition of the subunits. We can only state that the patterns resulting at the conditions stated here are not appreciably different from those which had been obtained with a lower voltage gradient and the final temperature of 45–50°C only.

If the duration was extended to 6 hours, the migrated distances increased proportionally and a good resolution was maintained, but not improved.

LENGTH OF DENATURATION

Engel (1962) showed that the denaturation of collagen proceeds in two stages: the helical structure is lost rapidly but the separation of the chains takes more time. The α- and β-components are not stable, but after 9 hours at 40°C at pH 3·7, 10–20 per cent of the β-component was lost and degradation products were obtained from both α- and β-fractions (Engel and Beier, 1963). The present studies were designated to check the electrophoretic pattern after various periods of denaturation (Hollmén and Kulonen, 1964a).

After initial denaturation at 40°C for 15 min the sample of acid-soluble rat tail tendon collagen was divided in separate lots, which were kept at 4°C until they were immersed in the 40°C water bath to get the desired denaturation periods from 30 min to 7 days at the beginning of the simultaneous electrophoretic run on a same gel sheet.

The slow-migrating fractions, which represent larger complexes, were lost during the denaturation of 1–2 days. The α1-band becomes rather broad, and on the basis of unpublished work from our laboratory we belive that it is heterogeneous (cf. Piez *et al.*, 1963). After a denaturation of 24 hours there appears a new distinct band, which migrates faster than the α2-component (*Figure* 2), and corresponds to the commercial gelatin from limed precursors (Näntö *et al.*, 1963).

ELECTROPHORESIS AT VARIOUS TEMPERATURES

The 'reconstituted' collagen at room temperature yields electrophoretic patterns, which differ from those obtained at +40°C (Näntö *et al.*, 1963). The result can be explained by the effect of the temperature during the run,

which was studied in more detail. The samples of acid-soluble rat tail tendon collagen were denatured at 40°C for 15 min. The temperature in the gel was stabilized efficiently by a continuous circulation of water from a thermostated vessel (Hollmén and Kulonen, 1964b).

At 34°C all the usual fractions migrated. At 30°C the *x*-bands were retained on the starting line, at 26°C migrated α2-, α1- and β1-units only (*Figure 2d*), at 22°C the α2- and α1-units, and at 18°C the α2-unit alone. The soluble collagen in the native state does not move in these conditions. The identification of the bands, which had been obtained at the lower temperatures, was ascertained by a repeated electrophoresis at 40°C. The refolding (von Hippel and Wong, 1963, Drake and Veis, 1964) of the different subunits does not seem to occur at the same temperature.

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APPENDIX. ELECTROPHORETIC FRACTIONATION OF TISSUE MUCOPOLYSACCHARIDES

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The electrophoresis on cellulose acetate sheets (Näntö, 1963) was applied on materials isolated from various sources (*Figure 4*). In many tissues we observed material which moves faster than the chondroitin sulphates (*Figure 4d*), and is destroyed by the action of desoxyribonuclease and absorbs ultra-violet light. The solubility of the cetylpyridinium chloride (CPC-) precipitate of this

fraction differed from the respective solubility of the acid mucopolysaccharides with similar migration rate (*Figure 4e*). The position of the keratosulphates in the fractionation pattern varies. In many preparations there appears a slowly migrating band (*e*), which has not been identified.

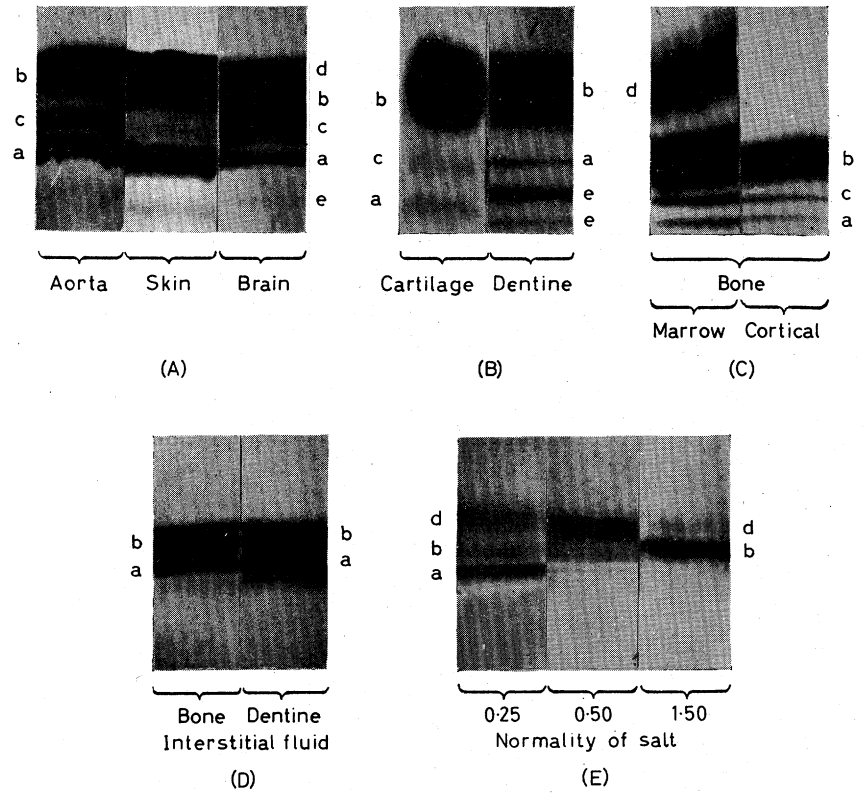

Figure 4. Acid mucopolysaccharides from various sources fractionated with electrophoresis on cellulose acetate sheet. (A–C). Comparison of various tissues; (D). Interstitial fluids, obtained by a high speed centrifugation; (E). CPC-precipitated mucopolysaccharides from skin dissolved into salt solutions of indicated concentrations. The materials had been digested with papain, precipitated with four-fold volume of ethanol, dissolved in water, the acid mucopolysaccharides precipitated again with CPC, dissolved in salt solution and finally precipitated with ethanol.

Identification: *a* hyaluronic acid, *b* chondroitin sulphates, *c* either heparitin sulphate, keratosulphate or chondroitin, *d* presumably nucleic acid, *e* non-identified fraction.

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